Kidney International, Vol. 66 (2004), pp. 1049–1060

Wegener's granulomatosis is associated with organ-specific antiendothelial cell antibodies

Carolina Holmén, Marta Christensson, Erna Pettersson, Johan Bratt, Pär Stjärne, Azza Karrar, and Suchitra Sumitran-Holgersson

Division of Clinical Immunology, Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden; Division of Renal Medicine, Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden; Division of Rheumatology, Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden; Division of Otorhinolaryngology, Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden; and Division of Transplantation Surgery, Huddinge University Hospital, Karolinska Institutet, Stockholm, Sweden

Wegener's granulomatosis is associated with organ-specific antiendothelial cell antibodies.

Background. Antiendothelial cell antibodies (AECA), usually detected using human umbilical vein endothelial cells (HUVEC), are frequently observed in systemic vasculitis, but their pathogenic role is unclear. Heterogeneity of endothelial cells necessitates use of clinically relevant endothelial cells for elucidation of the role of AECA in systemic vasculitis involving small blood vessels of specific organs.

Methods. Human endothelial cells were isolated from normal tissue specimens from the nose, kidney, lung, liver, and umbilical vein. Using flow cytometry, AECA were detected against both unstimulated and cytokine-stimulated [tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ)] endothelial cells. Functional capacity of AECA was determined by complement fixation assay. Sera from patients with Wegener's granulomatosis (16), limited Wegener's granulomatosis (8), renal limited disease (4), microscopic polyangiitis (MPA) (5), rheumatoid arthritis (10), and systemic lupus erythematosus (SLE) (9), and from healthy controls (20) were analyzed.

Results. Compared with controls (*1*) Wegener's granulomatosis is significantly associated with noncytotoxic AECA that selectively bind surface antigens on unstimulated nasal, kidney, and lung endothelial cells; (*2*) binding of Wegener's granulomatosis AECA to kidney and nasal endothelial cells in particular was lost upon treatment with IFN-γ and TNF-α; (*3*) the two cytokines per se were cytotoxic (30%) to nasal and lung endothelial cells and lysis was further increased (60%) by addition of systemic vasculitis serum; and (*4*) Wegener's granulomatosis serum caused agglutination of cytokine-stimulated nasal endothelial cells.

Conclusion. Based on these findings we suggest that AECA may be one factor involved in the initiation of Wegener's granulomatosis. Antigen identification and elucidation of the pathogenic roles of AECA and inflammatory cytokines in systemic vasculitis using these cells will be particularly important.

Key words: antiendothelial cell antibodies, Wegener's granulomatosis, microscopic polyangiitis, inflammatory cytokines, primary vasculitis.

Received for publication January 20, 2004
and in revised form March 1, 2004, and April 4, 2004
Accepted for publication April 16, 2004

Vasculitis is a term for a group of diseases that involve inflammation in blood vessel walls and forms the pathologic basis of a diverse group of individual disease entities. The primary systemic vasculitic diseases, Wegener's granulomatosis and microscopic polyangiitis (MPA), share many common features, including pulmonary capillaritis, pauci-immune focal necrotizing and/or crescentic glomerulonephritis, and circulating antineutrophil cytoplasmic antibodies (ANCA) [1, 2]. The presence of ANCA in a large proportion of patients with systemic vasculitis is well established, although their pathogenic significance still remains obscure [1–3]. In Wegener's granulomatosis patients, ANCA are usually directed against the enzyme proteinase 3 (PR3-ANCA), and in MPA patients against myeloperoxidase (MPO-ANCA) or PR3 [1, 2]. Because both diseases are highly associated with ANCA, they are sometimes categorized as ANCA-associated systemic vasculitides (AASV). Wegener's granulomatosis is a disease involving granulomatous inflammation, necrosis, and vasculitis that most frequently targets the upper respiratory tract (sinuses, nose, trachea), lower respiratory tract (lungs), and kidneys [1]. MPA is a necrotizing small-vessel vasculitis involving mainly the skin, lungs, and kidneys [2]. However, an important difference between the two diseases is the presence of granulomatous inflammation in Wegener's granulomatosis, which is not observed in MPA.

Due to the involvement of blood vessel inflammation in these diseases, interest was already focused on finding antibodies against endothelial cells. Antiendothelial cell antibodies (AECA) are another population of antibodies with high prevalence in the sera of systemic vasculitis patients. AECA have been shown to recognize

constitutive endothelial cell surface proteins [3–5], and to fluctuate with the clinical activity of the disease [3, 4]. Evidence exists that AECA from Wegener's granulomatosis sera can modulate endothelial cell function. Incubation of human umbilical vein endothelial cells (HUVEC) with AECA IgG from Wegener's granulomatosis patients up-regulated the expression of E-selectin, intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1/CD106) [6]. Expression of these surface molecules indicates endothelial activation, and leads to leukocyte adhesion and diapedesis during an inflammatory response. AECA bound to HUVEC also induced a parallel increase in cytokine [interleukin (IL)-1 and IL-6)] and chemokine [IL-8 and monocytes chemotactic protein-1 (MCP-1)] production that seems to support the up-regulation of the mentioned adhesion molecules in an autocrine fashion [6].

The pathogenic role of AECA is further highlighted by an experimental model of induced systemic vasculitis wherein mice immunized with IgG AECA from a patient with Wegener's granulomatosis displayed histopathologic signs of both renal and pulmonary vasculitis [7]. Furthermore, patients positive for AECA, but negative for ANCA, have been found to be at risk of a clinical relapse [3, 8]. Together, these data support a pathogenic role for AECA.

Endothelial cells are known to display a heterogeneous functional and phenotypic profile, depending on the anatomic origin and/or vessel type [9]. Therefore, it is reasonable to suppose that AECA vary in specificities (i.e., are directed against different target endothelial cell antigens), depending on the vascular origin. Accordingly, it has been shown that AECA can target microvascular or macrovascular endothelial cells [10–13]. So far, the role of AECA in Wegener's granulomatosis has been studied using HUVEC as target cells. Keeping in mind that it is the small blood vessels of the upper respiratory tract (sinuses, nose, ears, and trachea), the lungs, and the kidneys that are specifically attacked in Wegener's granulomatosis and MPA, we questioned the use of embryonic large blood vessel endothelial cells such as HUVEC. Thus, we chose instead to isolate endothelial cells from normal tissue specimens taken from the relevant organs of attack, namely nose, kidney, and lung for the detection of AECA in vasculitis sera. Immunoglobulin class and functional capacity of AECA to lyse these target cells by complement fixation were also determined.

METHODS

This study has been approved by the local Swedish Ethical Committee.

Monoclonal antibodies, reagents, and cells

Mouse anti-human monoclonal antibodies, unconjugated, fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated, were obtained as follows: CD106 (FITC), CD62E (PE), CD142, and CD144 (BD PharMingen, San Diego, CA, USA); CD105 (FITC), CD141, von Willebrand factor (FITC), and sheep antihuman IgA (FITC) (Serotec, Oxford, UK); CD31 (FITC) and *Ulex europaeus* (FITC) (Sigma-Aldrich, Munich, Germany); and acetylated low-density lipoprotein (Ac-LDL) (FITC) (Molecular Probes, Inc., Eugene, OR, USA). In addition, goat antihuman IgG and IgM $F(ab')_2$ fragment (FITC) and goat antimouse IgG (FITC) were purchased from Jackson ImmunoResearch (Baltimore, MD, USA); sheep antihuman IgG1, IgG2, IgG3, and IgG4 (all FITC) (The Binding Site, Birmingham, UK); goat antihuman IgE (FITC) (BioSource, Camarillo, CA, USA); mouse antihuman fibroblast and anti-alpha-actin antibodies (Harlan Sera-Lab, Loughborough, UK) and mouse antihuman epithelial antigen antibodies (Dakopatts AB, Älvsjö, Sweden). Recombinant human tumor necrosis factor-alpha (TNF-α) and interferon-gamma (IFN-γ) were purchased from R&D Systems (Abingdon, UK). Rabbit complement was from Cedarlane Laboratories Ltd. (Hornby, ON, Canada), and RPMI 1640 medium was from Gibco (Paisley, UK). Propidium iodide was from BD Pharmingen. Trypsin-ethylenediaminetetraacetic acid (EDTA), dispase, percoll, collagenase, and 4,6-Diamidino-2-phenylindole (DAPI) were from Sigma-Aldrich (Stockholm, Sweden).

Human kidney endothelial cells (HKEC), human nasal endothelial cells (HNEC), and human liver sinusoidal endothelial cells (HLSEC) were cultivated in endothelial selective medium MCDB 131 (Gibco, Gaithersburg, MD, USA) containing 10% heat-inactivated human AB serum, 5 mmol/L L-glutamine, and 100 μg/mL penicillin/streptomycin (PEST). The medium was further supplemented with endothelial cell growth medium (EGM-2) singlequots (Clonetics, BioWhittaker, Walkersville, WV, USA) (MCDB complete medium). Primary human microvascular endothelial cells from lung (HLMEC) and HUVEC (pooled from >three donors) were purchased from Clonetics and cultivated in recommended EGM-2-MV medium supplemented with EGM-2-MV singlequots.

Patients

Thirty-seven sera samples from 24 Wegener's granulomatosis patients, including patients with a limited form of Wegener's granulomatosis with only upper airway involvement and PR3-ANCA (mean age 68 years, 16 men and 12 women) and five patients with microscopic polyangiitis (MPA) and MPO-ANCA (mean age 81 years, two men and three women) were studied. Disease entities

Table 1. Some clinical and demographic data of vasculitis patients at debut (Group 1)

Patient number	Diagnosis	Gender	Age	Main organ involvement	Treatment	Disease activity
1[a]	LWG	F	52	ENT	No	Yes
2[a]	LWG	F	74	ENT	No	Yes
3[a]	WG	M	73	ENT, kidney	CS	Yes
4[a]	WG	M	59	ENT, kidney	CS	Yes
5[a]	WG	M	50	ENT, kidney	No	Yes
6[a]	WG	M	76	ENT, lung, kidney	No	Yes
7[a]	WG	M	35	ENT, eye, kidney	No	Yes
8[a]	RLD	M	73	Kidney	No	Yes
9[a]	WG	M	80	ENT, lung, kidney	No	Yes
10	WG	F	53	ENT, kidney	No	Yes
11	WG	M	82	Lung, kidney	No	Yes
12	WG	F	83	ENT, eye, CNS, kidney	CS	Yes
13	RLD	F	89	Kidney	No	Yes
14	RLD	F	76	Kidney	No	Yes
15	LWG	F	40	ENT	No	Yes
16	LWG	M	78	ENT	CS	Yes
17	LWG	M	87	ENT	No	Yes
18	RLD	F	64	Kidney	No	Yes
19	MPA	F	70	Lung, kidney	No	Yes
20	MPA	F	83	Lung, kidney	No	Yes
21	MPA	M	80	GI, kidney	No	Yes
22	MPA	F	86	Joints, kidney	No	Yes
23	MPA	M	87	Heart, kidney	No	Yes
In remission (group 2)						
1[a]	LWG	F	52	ENT	CS + CP	Low
2[a]	LWG	F	74	ENT	CS + CP	Low
3[a]	WG	M	73	ENT, kidney	CS + CP	No
4[a,b]	WG	M	59	ENT, kidney	CS	No
5[a]	WG	M	50	ENT, kidney	CS + Aza	No
6[a]	WG	M	76	ENT, lung, kidney	CS + CP	No
7[a]	WG	M	35	ENT, eye, kidney	CS + CP	No
8[a]	RLD	M	73	Kidney	CS + CP	No
9[a]	WG	M	80	ENT, lung, kidney	CS + Aza	No
24	LWG	F	29	ENT	CS + Mtx	No
25	WG	F	77	ENT, kidney	No	No
26	LWG	M	72	ENT, eye	CS + CP	No
27	WG	M	73	ENT, kidney	CS + Aza	No
28	LWG	M	74	ENT, eye	CP	No
In quiescence (group 3)						
29	WG	M	67	Eye, kidney	No	No
30	WG	M	82	ENT, eye, kidney	CP	Low
31	WG	M	60	ENT, kidney	MTx	No
32	WG	F	67	ENT, kidney	No	No
33	WG	F	84	ENT, eye, kidney	MTx	No

Abbreviations are: LWG, limited Wegener's granulomatosis; WG, Wegener's granulomatosis; RLD, renal limited disease; MPA, microscopic polyangiitis; ENT, ear, nose, throat; CS, corticosteroids; CP, cyclophosphamide; MTx, methotrexate; Aza, azathioprine; GI, gastrointestinal tract; CNS, central nervous system.
[a]Patients were reanalyzed while in remission; [b]Patient on dialysis.

were defined using the "Chapel Hill Consensus on the Nomenclature of Systemic Vasculitides" for Wegener's granulomatosis and MPA [14]. Renal limited disease ($N = 4$) included patients having pauci-immune necrotizing glomerulonephritis with or without crescents. Disease activity was defined according to the Birmingham vasculitis activity score (BVAS) [15]. The demographic and clinical characteristics of the vasculitis patients are presented in Table 1. These patients were divided into three groups. Group 1 contained 23 patients with newly diagnosed active disease. Blood samples were collected before initiation of immunosuppressive therapy. Group 2 contained 14 patients in clinical remission. Nine of the patients (Table 1) from group 1 were reanalyzed 3 to 11 months later while in remission, while five patients (24 to 28 in Table 1) were analyzed in clinical remission but not at time of admission. Group 3 contained five patients in quiescence (2 to 10 years after disease onset). For purpose of simplicity Wegener's granulomatosis, limited Wegener's granulomatosis, and renal limited disease will be referred to as Wegener's granulomatosis.

Ten patients with rheumatoid arthritis (mean age 59 years, four men and six women) and nine patients with systemic lupus erythematosus (SLE) (mean age 29 years, one man and eight women) were also included in the study. All rheumatoid arthritis and SLE patients were undergoing treatment.

Patient serum samples were separated from blood samples by centrifugation and stored at –20°C until use.

Serum from 20 age- and gender-matched healthy persons served as controls.

Isolation and cultivation of different target endothelial cells

Isolation of HNECs. HNECs were isolated from an approximately 1 cm nose biopsy specimen. After informed consent, patients scheduled for surgery due to structural nasal deformities, such as septal deviation, were selected. During the surgical procedure, tissue from the inferior turbinate was resected using a scalpel with an atraumatic technique to reduce the damage to the sample. The tissue was divided into small pieces (approximately 2 mm) and enzymatically digested using a 0.2% collagenase solution for 5 to 7 minutes in a 37°C water bath. These tissue samples were washed with MCDB 131 medium containing 5% fetal calf serum (FCS) (Gibco) and placed in 0.2% gelatin-coated wells of a 24-well culture plate. Each piece was placed in an individual well and incubated with the endothelial cell–selective medium MCDB 131 containing 2 ng/mL of vascular endothelial growth factor (VEGF). After 24 hours, the medium was further supplemented with 10% heat-inactivated human AB serum and EGM-2 singlequots. Cells were allowed to grow out from the nose explants. Areas with endothelial cell growth were manually picked and grown on separate culture plates. HNEC were cultured in MCDB 131 complete medium.

Isolation of human kidney and liver sinusoidal endothelial cells

Human kidney and liver endothelial cells were isolated from respective healthy organs (intended but not used for transplantation as no suitable recipient was found) according to a method described earlier [16, 17]. In short, tissue specimens were manually minced (into small cubes) and enzymatically digested with dispase (1.6 U/mL) overnight at 4°C. Thereafter, the individual tissue pieces were manually disaggregated with a flat instrument to release the endothelial cells. The decanted cell suspensions were centrifuged at 666*g* 4°C for 10 minutes and the organ-specific endothelial cells isolated on a density gradient of 35% percoll (Sigma Chemical Co., St. Louis, MO, USA) at 5000*g* 4°C for 10 minutes. The top band of the gradients was collected, the cells washed in phosphate-buffered saline (PBS), seeded on gelatin-coated tissue culture flasks and grown at 37°C in a humidified atmosphere of 5% CO_2 in air. In cultures where fibroblast contamination was observed, the endothelial cells were hand-picked for further culture. Cell monolayers were passaged at confluence using trypsin-EDTA. HKEC and HLSEC were routinely cultured in MCDB 131 complete medium.

HUVEC and HLMEC were cultured in medium recommended by the manufacturers.

Phenotyping of endothelial cells

Single-color fluorescence was used to phenotypically characterize HNEC, HKEC, HLSEC, HUVEC, and HLMEC. The procedure is described elsewhere [17]. Primary antibodies for staining were anti-CD141 (thrombomodulin), anti-CD142 (tissue factor), anti-CD144 (vascular endothelial-cadherin), anti-AcLDL, anti-*Ulex Europaeus* (lectin), anti-CD106 (VCAM-1), anti-CD62E (E-selectin), anti-CD31 [platelet-endothelial cell adhesion molecule (PECAM)], anti-von Willebrand factor, anti-CD105 (endoglin), anti-epithelial antigen, anti-fibroblast, and anti-alpha-actin (smooth muscle cell). The unconjugated primary antibodies were further stained with FITC-conjugated goat antimouse secondary antibodies. Intracellular staining for alpha-actin was performed using 5% saponin-treated endothelial cells as described earlier [18].

Screening of patient sera for antiendothelial cell antibodies using the flow cytometer. In all experiments, one set of cell samples remained untreated, while another set was stimulated for 16 hours with recombinant human IFN-γ (200 ng/mL) and TNF-α (20 ng/mL).

For the flow cytometric assay, unstimulated/stimulated HNEC/HLMEC/HKEC/HLSEC/HUVEC was used for the screening of AECA in sera of Wegener's granulomatosis, MPA, rheumatoid arthritis, SLE patients, and healthy controls. Sera from healthy nontransfused blood group AB males known not to have any antibodies served as negative controls. A pool of sera from patients who had formed alloantibodies as a result of multiple blood transfusions or organ transplantations was used as positive control. For the assay, 5×10^5 cells were incubated with 50 μL of patient serum for 1 hour at room temperature, and then washed twice with PBS. Ten microliters of 1:4 diluted fluoresceinated $F(ab')_2$ fragments of goat antihuman IgG (Fc-specific) and antihuman IgM (μ chain specific) antibodies were added and incubated at 4°C on ice in the dark for 30 minutes. The cells were washed, resuspended in 200 μL of PBS and immediately prior to analysis 4 μL of propidium iodide (50 μg/mL) was added to detect dead cells. Cells were then analyzed on a Becton Dickinson flow cytometer (FACSorter) (Becton Dickinson, Franklin Lakes, NJ, USA). Fluorescence signals from 10,000 cells were counted and the percentage of FITC-positive cells was recorded. A shift in the mean fluorescence of 20 channels in the test sample as compared to negative control was considered as positive, determined as described before [19].

The immunoglobulin class and subclass of AECA in six Wegener's granulomatosis sera giving a positive reaction with HNEC and HKEC were further defined using sheep antihuman IgG subclass specific IgG1, IgG2, IgG3, and IgG4 antibodies. Eight Wegener's granulomatosis patients with sufficient serum samples available were also tested for titers of AECA (1:10, 1:50, 1:100, and 1:200).

To determine whether the AECA in Wegener's granulomatosis sera were directed against blood group histocompatibility antigens, Wegener's granulomatosis sera were absorbed against a pool of packed red blood cells, platelets (express only class I histocompatibility antigens) and lymphocytes (express both class I and class II histocompatibility antigens). For this purpose, 50 μL of patient serum was added to pooled and packed red blood cells and incubated overnight at 4°C. The cells were centrifuged and the serum was separated. The serum was subsequently absorbed using a similar procedure against pooled and packed platelets as well as 1×10^6 pooled lymphocytes. The absorbed sera were once again tested with HNEC, HKEC, and HLMEC for reactivity as described above. Furthermore, reactivity of unabsorbed Wegener's granulomatosis sera to a monocytic cell line, U937 (ATCC, Rockville, MD, USA) and a human renal epithelial primary cell line (HRE) (Clonetics) was also performed to test for cross-reactivity with monocytic or epithelial antigens.

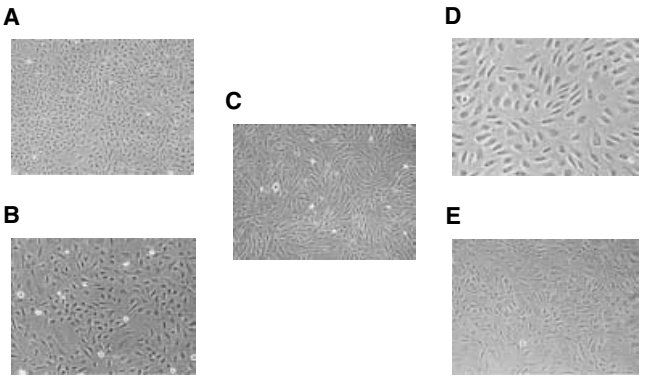

Fig. 1. Morphologic characteristics of the isolated endothelial cells. Morphology of primary (*A*) human nasal endothelial cells (HNEC), (*B*) human kidney endothelial cells (HKEC), (*C*) human liver sinusoidal endothelial cells (HLSEC), (*D*) human umbilical vein endothelial cells (HUVEC), and (*E*) human microvascular endothelial cells from lung (HLMEC) using light microscopy. All cell types showed contact inhibition, and HNEC, HUVEC, and HLMEC showed cobblestone morphology characteristic of endothelial cells, while HKEC and HLEC were more spindle-shaped.

Immunocytochemistry

Tissue culture plates with a 0.2% gelatin coating were used to grow 3×10^5 HNEC and HKEC. Cells were allowed to attach (24 hours) prior to staining. Cells were left either untreated or stimulated with IFN-γ and TNF-α for 16 hours. For immunocytochemistry, cells were washed twice with PBS and incubated with either Wegener's granulomatosis or MPA or healthy control sera diluted 1:2 (in RPMI 1640 medium) at room temperature for 3 hours. The secondary antibodies used were either FITC-conjugated goat antihuman IgG or IgM diluted 1:500 (in PBS). After incubation for 1 hour at 4°C on ice, the cells were washed twice with PBS and analyzed under a fluorescence microscope.

Microcytotoxicity assay

The flow cytometric analysis demonstrated that in most cases the binding of vasculitis AECA was observed with only unstimulated but not cytokine-stimulated HKEC and HNEC. Therefore, detection of the functional capacity of AECA in vasculitis sera using the microcytotoxicity assay was performed with only unstimulated HKEC and HNEC. The method used was as described earlier [20]. Briefly, the cells were harvested with trypsin-EDTA, washed once with PBS, and resuspended in culture medium at a concentration of 3 to 4×10^6 cells/mL. Prior to addition of 0.5 μL unstimulated HKEC or HNEC, 0.5 μL of vasculitis sera yielding positive reactions in the flow cytometric analysis were each added to microcytotoxicity plates in triplicates. Cells and sera were incubated for 1 hour at room temperature. Two microliters of rabbit complement containing the dyes acridine orange and ethidium bromide were added. After 45 minutes incubation at room temperature, the reactions were read in a fluorescence microscope. Reactions were considered positive when there was lysis of more than 10% above background as compared to the negative control. Negative controls consisted of heat-inactivated serum from normal healthy individuals. In some cases, the vasculitis sera were inactivated for 1 hour at 56°C to determine the role of complement factors in the cytotoxic process.

Statistical analysis

Chi-square test and, when appropriate, Fisher's exact test were used to compare categorical parameters. Differences were considered significant if $P < 0.05$.

RESULTS

Culture and morphologic characterization of human nasal, kidney, and liver endothelial cells

Following isolation, HNEC grew in several clusters that formed monolayers within 4 to 5 days, and showed contact inhibition and the cobblestone morphology characteristic of endothelial cells (Fig. 1A). Within 10 days the HNEC became confluent. We have previously isolated and used well-characterized HLSEC at our laboratory [17]. In the present study, when HKEC or HLSEC were isolated and plated in gelatin-coated flasks, small adherent clusters of two to five confluent, cobblestone- and some stellate to fusiform-shaped cells were noted within 12 to 18 hours. After about 1 week, these clusters enlarged until they touched and formed a confluent layer (Fig. 1B and C). HNEC could be maintained with stable morphology and growth rate for approximately 10 passages and HKEC and HLSEC for approximately 18 passages, after which the cells ceased to divide. HUVEC and HLMEC also

Table 2. Phenotypic characteristics of endothelial cells isolated from human umbilical vein (HUVEC), kidney (HKEC), nose (HNEC), lung (HLMEC), and liver (HLSEC)

Antibodies	HUVEC% cells expressing marker	HKEC% cells expressing marker	HNEC% cells expressing marker	HLMEC% cells expressing marker	HLSEC% cells expressing marker
CD 141	+ (99%)	+ (98%)	+ (95%)	+ (96%)	+ (100%)
CD 142[a]	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
CD 144	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (89%)
Ac-LDL	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Ulex Europaeus	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
CD 106[a]	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
CD 62E[a]	+ (73%)	− (0%)	− (0%)	+ (68%)	− (0%)
CD31	+ (100%)	− (0%)	− (0%)	+ (100%)	− (0%)
von Willebrand factor	+ (98%)	+ (95%)	+ (96%)	+ (98%)	− (0%)
CD105	+ (100%)	+ (100%)	+ (100%)	+ (100%)	+ (100%)
Epithelial antigen	− (0.1%)	− (0.05%)	− (0.05%)	− (0.1%)	− (0%)
Fibroblasts	− (0.005%)	− (0.005%)	− (0.05%)	− (0.01%)	− (0.01%)
Alpha-actin	− (0%)	− (0%)	− (0%)	− (0%)	− (0%)

Ac-LDL is acetylated low-density lipoprotein.
[a]Expressed only activated endothelial cells.

Table 3. Number and percentage of patients and healthy controls with antibodies against the tested endothelial cells

Patients (number)	HUVEC		HKEC		HNEC		HLMEC		HLSEC	
	US	S[a]	US	S[a]	US	S[a]	US	S[a]	US	S[a]
WG (28)	2 (7%)	3 (11%)	20 (71%)	8 (29%)	17 (61%)	3 (11%)	7 (25%)	12 (43%)	0 (0%)	0 (0%)
MPA (5)	0 (0%)	1 (20%)	3 (60%)	0 (0%)	4 (80%)	0 (0%)	3 (60%)	4 (80%)	1 (20%)	2 (40%)
RA (10)	0 (0%)	0 (0%)	1 (10%)	1 (10%)	1 (10%)	1 (10%)	1 (10%)	6 (60%)	0 (0%)	1 (10%)
SLE (9)	9 (100%)	9 (100%)	7 (77%)	7 (77%)	7 (77%)	7 (77%)	5 (55%)	5 (55%)	6 (67%)	6 (67%)
HC (20)	1 (5%)	1 (5%)	1 (5%)	1 (5%)	1 (5%)	1 (5%)	5 (25%)	6 (30%)	0 (0%)	0 (0%)

Abbreviations are: WG, Wegener's granulomatosis; MPA, microscopic polyangiitis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; HC, healthy controls; HUVEC, human umbilical vein endothelial cells; HKEC, human kidney endothelial cells; HNEC, human nasal endothelial cells; HLMEC, human lung microvascular endothelial cells; HLEC, Human liver sinusoidal endothelial cells; S, stimulated; US, unstimulated.
[a]Cells were stimulated for 16 hours with interferon-gamma (INF-γ) (200 ng/mL) and tumor necrosis factor-alpha (TNF-α) (20 ng/mL).

showed the typical cobblestone morphology of endothelial cells (Fig. 1D and E).

Phenotypic heterogeneity of human nasal, kidney, lung, liver, and umbilical vein endothelial cells

Table 2 shows the differences in the phenotypic characteristics between the various primary endothelial cell lines. HNEC, HKEC, and HLSEC did not express the activation marker E-selectin (CD62E) even at 6 and 8 hours after activation, nor did they express the adhesion molecule PECAM (CD31). However, all endothelial cells expressed other activation markers VCAM-1 and CD142. In addition, HLSEC did not express the endothelial cell associated marker von Willebrand factor confirming our previous studies [17].

A high frequency of Wegener's granulomatosis patients have AECA against human nasal, kidney, and lung endothelial cells

A significantly higher fraction of Wegener's granulomatosis patients had AECA that bound to unstimulated HNEC (17/28) (61%) and HKEC (20/28) (71%), and cytokine-stimulated HLMEC (12/28) (43%) as compared to HUVEC (3/28) (10%) or HLSEC (0/28) ($P < 0.0001$, $P < 0.0001$, and $P = 0.008$, respectively) (Table 3). In addition, MPA patients showed a similar pattern of reactivity to the same target endothelial cells as Wegener's granulomatosis. This organ-specific pattern of reactivity was not observed with sera from patients with rheumatoid arthritis or SLE. Surprisingly, a high fraction of rheumatoid arthritis patients reacted strongly with only stimulated HLMEC, while SLE sera reacted with all target endothelial cells (Table 3). Reactivity to HLMEC was in general high in all patient groups studied, including controls. The pattern of AECA reactivity to the various target endothelial cells in Wegener's granulomatosis and MPA patients is shown in Table 4. Three fourths (75%) of renal limited disease patients had AECA only reactive with HKEC. Five eighths (62.5%) of patients with Wegener's granulomatosis limited to the upper respiratory tract had AECA against HNEC and/or HLMEC, while three eighths (38%) of the same patients also had AECA against HKEC. In addition, 13/16 (81%) of Wegener's granulomatosis patients with kidney involvement had AECA reactivity against HKEC. Thus, in most cases the pattern of AECA reactivity correlated well with the clinical organ involvement.

We found that Wegener's granulomatosis sera positive for AECA with HNEC, HKEC, and HLMEC when

Table 4. Antineutrophil cytoplasmic antibodies (ANCA) and antiendothelial cell antibodies (AECA) in vasculitis patients at debut (group 1)

Patient number	ANCA			AECA		
	PR3/MPO *U/mL*	HNEC	HKEC	HLMEC	HUVEC	HLSEC
1[a]	35/PR3	–	–	+	+	–
2[a]	65/PR3	+	+	+	–	–
3[a]	290/PR3	+ (1:100)	+ (1:200)	+	–	–
4[a]	120/PR3	–	–	–	–	–
5[a]	10/PR3	+	–	+	–	–
6[a]	45/PR3	+ (1:200)	+ (1:200)	+	–	–
7[a]	100/PR3	+ (1:100)	+ (1:200)	–	–	–
8[a]	105/MPO	+	+	–	–	–
9[a]	20/MPO	+	+	+	–	–
10	100/PR3	+	+	–	–	–
11	35/MPO	+ (1:100)	+ (1:200)	–	–	–
12	80/MPO	–	+	+	–	–
13	90/MPO	–	+	–	–	–
14	500/MPO	–	+	–	+	–
15	24/PR3	+	+	+	–	–
16	50/PR3	+	–	+	+	–
17	15/MPO	+	–	+	–	–
18	45/PR3	–	+	–	–	–
19	80	+	–	+	–	–
20	150	–	+	+	–	–
21	300	+	+	+	–	–
22	125	+	–	–	–	–
23	75	+	+	+	–	–
In remission (group 2)						
1[a]	20/PR3	–	–	+	+	–
2[a]	25/PR3	+	+	+	–	–
3[a]	85/PR3	+ (1:50)	+ (1:50)	+	–	–
4[a]	25/PR3	–	–	–	–	–
5[a]	<10/PR3	+	–	+	–	–
6[a]	<10/PR3	+ (1:50)	+ (1:100)	+	–	–
7[a]	<10/PR3	+ (1:50)	+ (1:100)	–	–	–
8[a]	<10/MPO	–	–	–	–	–
9[a]	<10/MPO	–	+	–	–	–
24	15/PR3	+	+	–	–	–
25	25/MPO	+ (1:50)	+ (1:50)	–	–	–
26	<10/PR3	–	–	–	–	–
27	<10/PR3	–	+	–	–	–
28	<10/PR3	–	–	–	–	–
In quiescence (group 3)						
29	210/PR3	+	+	+	–	–
30	80/PR3	+ (1:50)	+ (1:50)	–	–	–
31	<10/PR3	–	–	–	–	–
32	<10/MPO	+ (1:10)	+ (1:10)	+	–	–
33	95/PR3	–	+	–	–	–

Abbreviations are: HNEC, human nasal endothelial cells; HKEC, human kidney endothelial cells; HLMEC, human lung microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; HLSEC, human liver sinusoidal endothelial cells; PR3, proteinase 3; MPO, myeloperoxidase.

[a]Sera from these patients were analyzed at debut and during remission.

absorbed against red blood cells, platelets, and lymphocytes retained reactivity to the endothelial cell lines even after absorption (Fig. 2A). In addition, no reactivity to the monocytic cell line U937 or renal epithelial cell line was observed (Fig. 2B).

The detection of ANCA and AECA in the vasculitis patients is shown in Table 4. All (5/5) newly diagnosed MPA patients had AECA against either HNEC and/or HKEC and/or HLMEC. Furthermore, 10/19 (53%) of patients in remission or quiescence had no detectable ANCA (<10 units/mL) (Table 4). However, 8/10 (80%) of these patients had AECA. Thus, a high number of treated vasculitis patients continued to have AECA, which is similar to the results obtained with the treated SLE patients in this study (Table 3). In contrast, the frequency of AECA reacting with HKEC and HNEC in all treated rheumatoid arthritis patients was significantly lower as compared to vasculitis patients ($P = 0.01$ and $P = 0.006$, respectively, Table 3).

Interestingly, the binding of Wegener's granulomatosis and MPA AECA to HNEC and HKEC was significantly lost upon cytokine stimulation with IFN-γ and TNF-α as compared to unstimulated cells ($P = 0.002$ and $P = 0.0003$, respectively) (Table 3). Similar results were obtained when cells were stimulated with either of the cytokines (data not shown); however, a greater decrease in binding was observed when both cytokines were added in combination. The loss of AECA binding

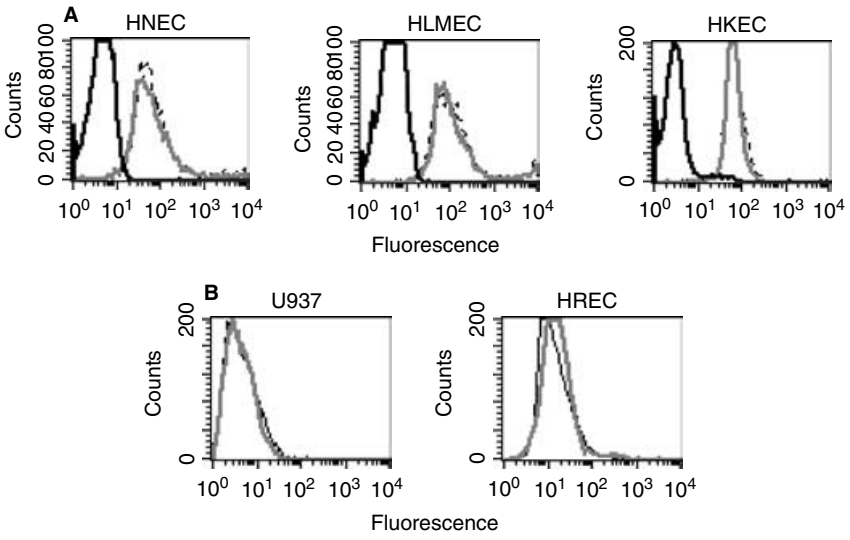

Fig. 2. Reactivity of sera containing endothelial cell reactive antibodies after absorption with various target cells. Incubation of unstimulated primary human nasal (HNEC), kidney (HKEC), and lung microvascular endothelial cells (HLMEC) with sera from Wegener's granulomatosis patients after absorption with pooled packed red blood cells, lymphocytes, platelets showed a positive reaction (*A*). This indicated that the antibodies in the Wegener's granulomatosis sera were not directed against blood group or histocompatibility antigens. In addition no cross-reactivity of Wegener's granulomatosis sera to a monocytic cell line U937 and a renal epithelial primary cell line was observed (*B*). Representative result from a single Wegener's granulomatosis patient is shown. Antibody binding before absorption (gray line), and after absorption (dashed line) is shown. Negative control is represented in black.

upon cytokine stimulation was not observed with either rheumatoid arthritis or SLE sera.

Sera from six Wegener's granulomatosis patients were chosen to identify the immunoglobulin class and titers of the AECA binding to unstimulated HNEC and HKEC. Using HNEC as target cells, we found that Wegener's granulomatosis sera contained a mixture of AECA that belonged to IgM, IgG, and IgA classes. However, using HKEC, 6/6 Wegener's granulomatosis patients had AECA that belonged to IgG4 and 5/6 also had IgM antibodies. The titers of AECA using HNEC and HKEC in sera of Wegener's granulomatosis patients with active disease was in the range of 1:100 to 1:200, respectively, while patients in remission and quiescence had AECA titers of 1:50 and 1:10, respectively (Table 4).

AECA in systemic vasculitis sera bind to cell surface antigens expressed on human nasal and kidney endothelial cells

Staining of HNEC and HKEC by immunocytochemistry for the binding of systemic vasculitis AECA showed that these antibodies bind to cell surface antigens expressed on these target cells (Fig. 3). No green fluorescence staining was observed when these cells were stimulated with IFN-γ and TNF-α, indicating once again the loss/modification of the antigen(s) on the cell surface. Sera from normal controls did not bind to these target endothelial cells.

Cytokines IFN-γ and TNF-α have a cytotoxic effect on human nasal and lung endothelial cells

The cytokines IFN-γ and TNF-α per se killed in general approximately 30% HNEC and 40% HLMEC as compared to HKMEC (18%), HLSEC (10%), and HUVEC (12%) [HNEC and HLMEC vs. HKMEC) ($P < 0.0001$), HNEC and HLMEC vs. HLSEC ($P < 0.0001$), HNEC and HLMEC vs. HUVEC ($P < 0.0001$)]. The percentage of cell death in the various unstimulated endothelial cell type is shown in Figure 4A. Importantly, addition of Wegener's granulomatosis serum to cytokine-stimulated HNEC and HLMEC further increased the lysis to approximately 60% as compared with unstimulated cells (30%) (Fig. 4). Even more important, the increased lysis of stimulated HNEC was specifically observed with only serum from the Wegener's granulomatosis or MPA patients and not from rheumatoid arthritis or SLE patients, while increased lysis of stimulated HLMEC was observed with sera from all groups of patients (Fig. 4B). On the other hand, addition of Wegener's granulomatosis or MPA serum to cytokine-stimulated HKEC, HLEC, and HUVEC did not increase cell lysis (Fig. 4B). Cell death was detected by the addition of propidium iodide using flow cytometric analysis. An increased cytotoxicity was observed when both cytokines were added in combination as compared to either of the cytokines (data not shown).

AECA do not lyse human nasal, kidney, or lung endothelial cells in a complement-dependent cytotoxic assay

We found that Wegener's granulomatosis sera both in the presence or absence of complement did not display any significant cytotoxicity toward unstimulated HNEC or HKEC. In 7/17 and 2/4 Wegener's granulomatosis and MPA patients, AECA killed 10% to 15% (see above) of unstimulated HNEC, respectively, while 7/20 and 2/3 Wegener's granulomatosis and MPA patients had cytotoxic AECA (10% to 15%) against HKEC, respectively, in the presence of rabbit complement as compared to negative controls. Live cells were visualized as green fluorescence and dead cells as red (Fig. 5). As stated above, cytokine

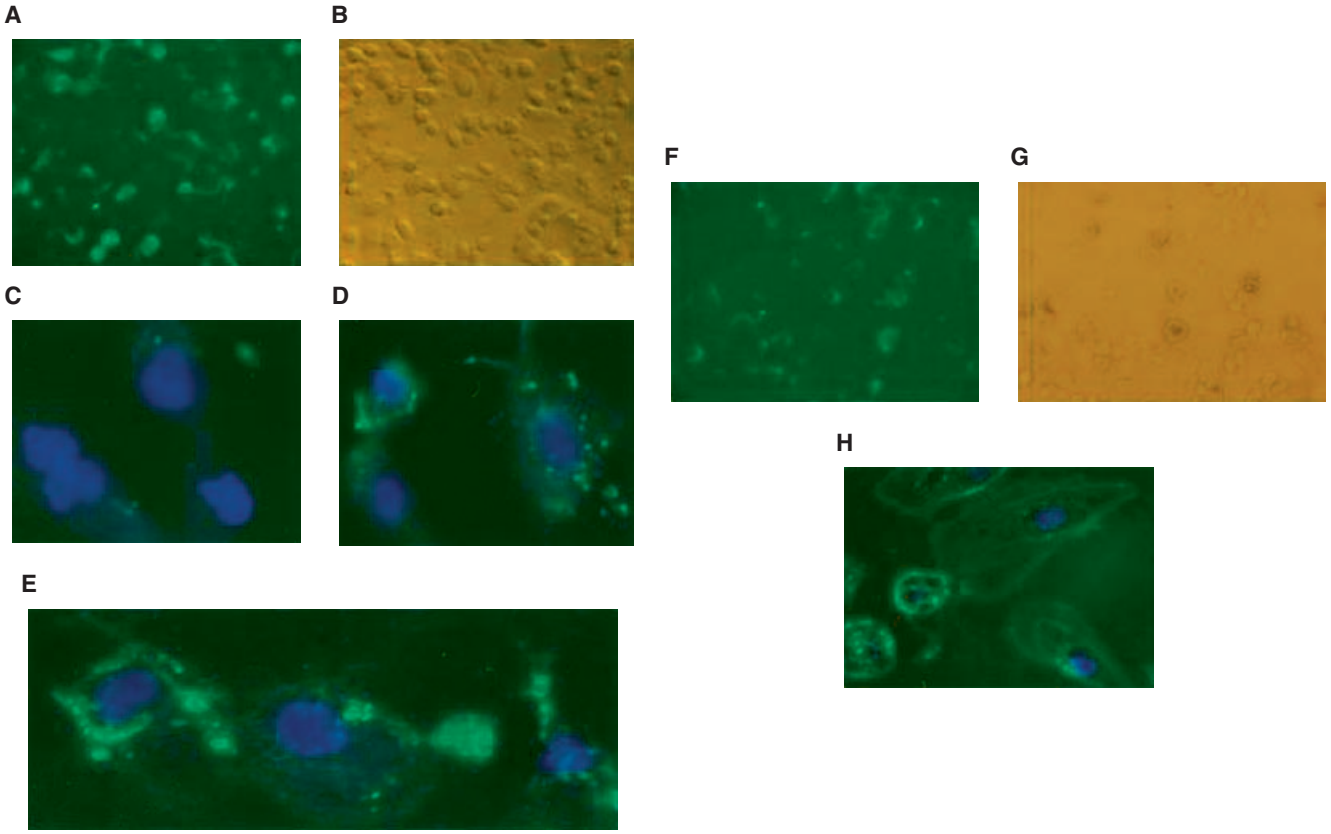

Fig. 3. Endothelial cell reactive antibodies binding to cell surface antigens. Incubation of unstimulated primary human nasal (HNEC) and kidney (HKEC) endothelial cells with sera from systemic vasculitis patients showed a positive surface fluorescent staining. Representative result from a single Wegener's granulomatosis patient is shown [(*A*) using fluorescence microscopy, (*B*) same field under light microscopy; 10×]. No positive staining with negative control serum (AB serum) was observed (*C*), while positive staining was observed with the positive control serum (pooled serum from alloimmunized patients) (*D*) and Wegener's granulomatosis serum (*E*). Cells under higher magnification 40× (C to E). The nucleus is stained blue using DAPI. Representative result from a single Wegener's granulomatosis patient using HKEC as target cells is shown [(*F*), using fluorescence microscopy, (*G*) same field under light microscopy; 10×]. (*H*) Cells under higher magnification 40×. The nucleus is stained blue using DAPI. DAPI, 4,6-Diamidino-2-phenylindole.

stimulation per se killed approximately 30% of HNEC (Fig. 5E) as compared to untreated HNEC (Fig. 5A). Interestingly, addition of either MPA or Wegener's granulomatosis serum resulted in increased lysis (approximately 40% and 60%, respectively) (Fig. 5F and G). Inactivation of Wegener's granulomatosis or MPA sera at 56°C did not decrease cell lysis of cytokine-stimulated HNEC (Fig. 5H) or HLMEC. Addition of positive Wegener's granulomatosis serum to cytokine-stimulated HNEC resulted in strong agglutination of the cells. However, no agglutination of stimulated HNEC was observed with MPA serum.

DISCUSSION

Our results demonstrate for the first time that a significantly high fraction of Wegener's granulomatosis patients had AECA against human nose, kidney, and lung endothelial cells but not HUVEC, confirming our hypothesis that HUVEC may not always be the suitable target in AECA assays. Thus, the heterogeneity of endothelial cells from various vascular beds demonstrates that the use of HUVEC to investigate mechanisms of injury in small-vessel vasculitis may be both inappropriate and misleading. The high frequency of AECA found in vasculitis sera using HUVEC, as reported in previous studies, was not reproducible in our study. In most, though not all, studies [21–25], the binding of AECA to HUVEC has been demonstrated using formalin- or glutaraldehyde-fixed cells. Fixation of cells increases both nonspecific binding and artifacts, which may give false positive results [26–28]. In the present study, we used unfixed viable endothelial cells. Furthermore, a pooled fraction of HUVEC from different individuals was used to eliminate individual differences.

It is well known that endothelial cells have specialized functions, depending on the tissue in which they are located. Vascular endothelium is characterized by heterogeneity in morphologic/functional aspects, marker proteins of cell activation, and responsiveness to cytokines

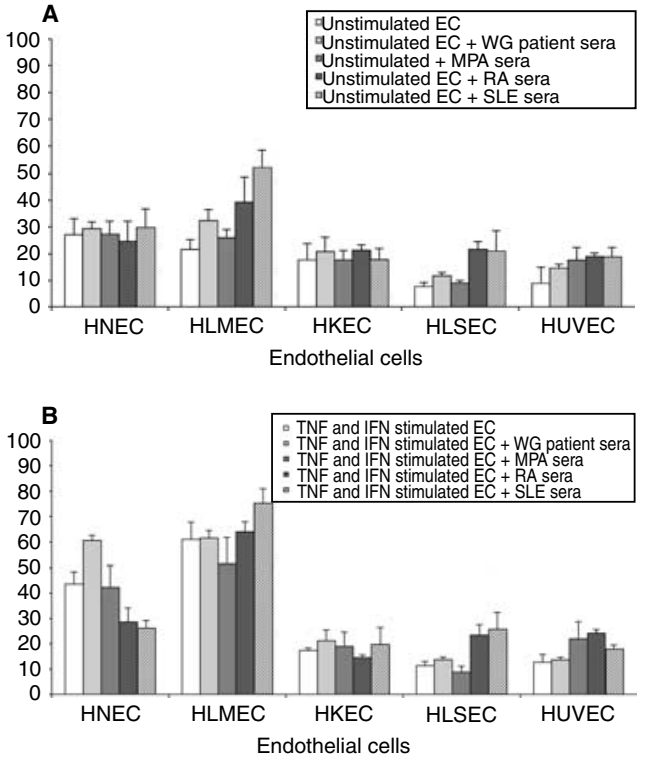

Fig. 4. Endothelial cell lysis by inflammatory cytokines and effect of serum addition from various patient groups on these cells. Cell lysis of unstimulated human nasal (HNEC), lung microvascular (HLMEC), kidney (HKEC), liver sinusoidal (HLSEC), and umbilical vein endothelial cells (HUVEC) and the same cells treated with sera from Wegener's granulomatosis (WG), microscopic polyangiitis (MPA), rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) patients is shown (*A*). HNEC (30%) and HLMEC (40%) were found to be significantly more susceptible to the cytotoxic effects of interferon-gamma (INF-γ) (200 ng/mL) and tumor necrosis factor-alpha (TNF-α) (20 ng/mL) per se as compared to HKEC (18%), HLSEC (10%), and HUVEC (12%) ($P < 0.0001$, $P < 0.0001$, $P < 0.0001$, respectively) (*B*). Addition of serum from patients with WG, MPA, RA, SLE to cytokine-treated HNEC and HLMEC resulted in increased cell lysis, indicating that cytokine stimulation may result in modulation of these cells, making them further susceptible to reactive components present in serum. The same was not observed with HKEC, HLSEC and HUVEC. Cell death was detected by the addition of propidium iodide using the flow cytometric analysis. Note the increased cell lysis of stimulated-HNEC observed with only systemic vasculitis sera, indicating a disease-specific phenomenon. The same was not observed with HLMEC.

[29, 30]. All the endothelial cells isolated in the present study expressed most of the endothelial-specific cell surface markers; however, some differences were observed, for example, the lack of the adhesion molecule E-selectin and PECAM expression on nose, kidney, and liver endothelial cells but not on lung and umbilical vein endothelial cells. Sinusoidal endothelial cells of the liver are well established as cells lacking the expression of PECAM and E-selectin [17]. Thus, our results showed both morphological and phenotypic differences between the EC from various organs reflecting the vast heterogenity observed in these cells.

In the present study, two interesting observations were made regarding the role of AECA: (*1*) the binding of systemic vasculitis AECA to cell surface antigens expressed on viable, unstimulated HNEC, HKEC, and HLMEC but not on HUVEC or HLSEC suggests that these antibodies are organ-specific and (*2*) AECA were not lytic, either in patients with clinical activity or quiescence. Reactivity of Wegener's granulomatosis AECA toward HNEC, HKEC, and HLMEC was not affected even after absorption of Wegener's granulomatosis sera with packed and pooled red blood cells, lymphocytes, and platelets indicating that antibodies are not directed to blood group or histocompatibility antigens. AECA have been reported to cross-react with monocytes [31–33] and therefore we tested Wegener's granulomatosis sera for cross-reactivity to a monocytic cell line U937 and a renal epithelial cell line and found no binding of Wegener's granulomatosis AECA with either of the two cell lines. The exact specificity of the antigen(s) recognized by AECA will be important. Interestingly, the same profile of AECA persisted despite remission; however, the titers of AECA were much lower during remission. Since the numbers of patients studied were small, we are currently performing a study with a larger number of Wegener's granulomatosis patients to confirm the shift in titers of AECA during remission.

Our findings suggest that AECA may be one of the several factors involved in the initiation of Wegener's granulomatosis but may not be directly involved in destruction of the vessel walls. The organ-specific binding of AECA in Wegener's granulomatosis to endothelial cells of the nose, lung, and kidneys may not result in lysis but instead directly trigger local production of inflammatory cytokines or alternatively might be instrumental in inducing endothelial phenotype changes [6, 17], resulting in inflammation or facilitation of cellular infiltration. This still remains to be proven and such studies are ongoing at our center. In addition, two important observations were made regarding the possible role of the inflammatory cytokines IFN-γ and TNF-α in Wegener's granulomatosis. First, treatment of HNEC and HKEC with IFN-γ and TNF-α induced a loss, or alteration, in the structure or accessibility of the endothelial cell surface molecules resulting in significantly decreased binding of Wegener's granulomatosis AECA to these stimulated cells. This finding may explain the reason why despite the reports of high levels of circulating AECA, Wegener's granulomatosis are characterized by the in vivo absence of a clear antibody deposit in the affected vessel wall [34]. Downregulation of the antigen(s) recognized by AECA after cytokine stimulation has also been described in hemolytic uremic syndrome (HUS) [35]. However, the antigen has not yet been identified.

Second, IFN-γ and TNF-α per se were cytotoxic to HNEC and HLMEC but not to HUVEC, HKEC, and

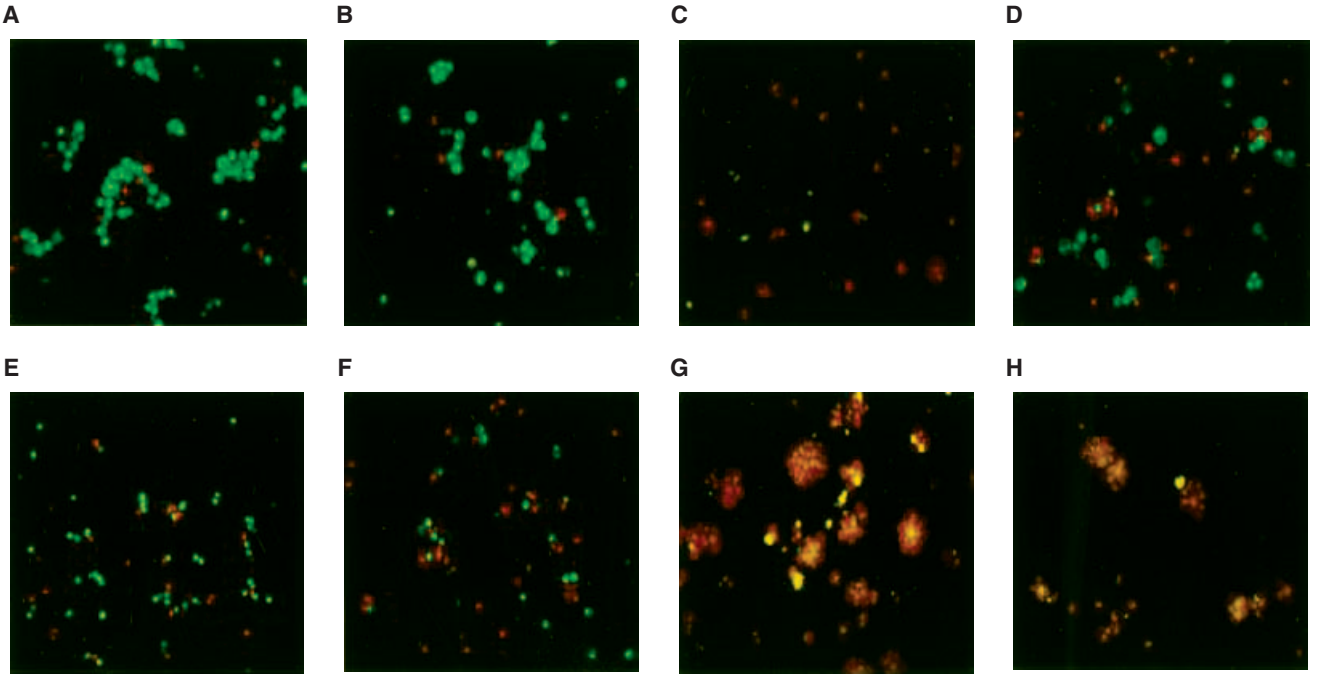

Fig. 5. Detection of functional capacity of antiendothelial cell antibodies (AECA) in a complement-dependent cytotoxic assay. AECA in sera from Wegener's granulomatosis patients did not lyse unstimulated human nasal (HNEC) or kidney (HKEC) endothelial cells in a complement-dependent cytotoxic assay. A representative result from one Wegener's granulomatosis patient using HNEC as target cells is shown in (*D*). This patient had AECA that killed 10% to 15% unstimulated HNEC in the presence of rabbit complement as compared to negative control. Live cells are visualized as green fluorescence (*A* and *B*) and dead cells as red (*C*, *F* to *H*). Interferon-gamma (INF-γ) and tumor necrosis factor-alpha (TNF-α) stimulation per se killed approximately 30% HNEC (*E*) as compared to untreated HNEC (A). Interestingly, addition of microscopic polyangiitis (MPA) (F) and Wegener's granulomatosis (G) serum resulted in increased lysis (approximately 60%). Addition of Wegener's granulomatosis (G) but not MPA (F) serum to stimulated HNEC resulted in strong agglutination of the cells. Inactivation of Wegener's granulomatosis sera at 56°C did not decrease cell lysis (H), indicating that complement components may not play a role in cell death.

HLSEC. Both cytokines have been shown to be cytotoxic to vascular endothelial cells [36, 37]. Our initial studies show that cell death induced by these cytokines may not occur via apoptosis, since the cells did not stain with annexin v but only with propidium iodide even at 4 hours after cytokine stimulation (preliminary results). We are currently investigating the reason why specifically HNEC and HLMEC are susceptible to the cytotoxic effects of IFN-γ and TNF-α. These observations are intriguing and it would be interesting to test using a larger group of patients, whether these cytokines may be directly involved in initiating destruction of the blood vessels in the lungs and nose of Wegener's granulomatosis patients.

Addition of Wegener's granulomatosis and MPA sera to cytokine-stimulated HNEC and HLMEC further increased cell lysis, indicating that cytokine stimulation may result in modulation of these cells, making them further susceptible to reactive components of Wegener's granulomatosis and MPA sera. Identification of the serum components in Wegener's granulomatosis that may enhance lysis of cytokine-stimulated HNEC and HLMEC will be important. Heat-inactivation of Wegener's granulomatosis serum did not result in decreased cytotoxicity, demonstrating that complement components probably do not play a role in the lytic process, since complement factors are heat labile and are inactivated at 56°C. Furthermore, increased cell lysis of stimulated HNEC was observed specifically with Wegener's granulomatosis and MPA sera, but not with sera from rheumatoid arthritis and SLE patients, reflecting a disease-specific phenomenon in systemic vasculitis. Increased lysis of stimulated HLMEC was observed with sera from all groups of patients, indicating differences in sensitivity to the effects of IFN and TNF by various endothelial cells. Interestingly, addition of Wegener's granulomatosis but not MPA serum to cytokine-stimulated HNEC resulted in strong agglutination of these cells. However, it is not possible to draw any conclusions due to the small numbers of patients studied.

CONCLUSION

We show that (*1*) that AECA react better with certain adult endothelial cells than with HUVEC; (*2*) that the reactivity appears to show some correlation with the organ distribution of disease shown clinically; and (*3*) cytokine pretreatment alters both the percentage of binding and the pattern on immunofluorescence. Thus, using unique clinically relevant target endothelial cells, we report important findings that could lead to a better understanding

of the complex pathological processes involved in Wegener's granulomatosis.

ACKNOWLEDGMENTS

We wish to thank Professor Erna Möller for financial support, Dr. Zhong He and Dr. Makiko Kumagai-Braesch for help with microscopy, and Dr. Grzegorz Nowak for statistical assistance. The present study was financed by grants from the the Medical Research Council, grant no. 00793 to Professor Erna Möller, and no. K2002-06X-14004-02B to Dr. Holgersson, The Konung Gustaf V's 80 Year Foundation, the Reumatikerförbundet Foundation, and the Sunds Foundation.

Reprint requests to Dr. Suchitra Sumitran-Holgersson, Department of Transplantation Surgery B56, Huddinge University Hospital, S-141 86 Stockholm, Sweden.
E-mail: suchitra.holgersson@cfss.ki.se

REFERENCES

1. Russell KA, Wiegert E, Schroeder DR, *et al*: Detection of antineutrophil cytoplasmic antibodies under actual clinical testing conditions. *Clin Immunol* 103:196–203, 2002
2. Day C, Savage C: Primary systemic vasculitis. *Minerva Medica* 92:349–363, 2001
3. Hoffman G, Specks U: Antineutrophil cytoplasmic antibodies. *Arthritis Rheum* 41:1521–1537, 1998
4. Del Papa N, Comforti G, Gambini D, *et al*: Characterization of the surface proteins recognized by anti-endothelial antibodies in primary autoimmune vasculitis. *Clin Immunol Immunopathol* 70:211–216, 1996
5. Gobel U, Eichhorn J, Kettritz R, *et al*: Disease activity and autoantibodies to endothelial cells in patients with Wegener's granulomatosis. *Am J Kidney Dis* 28:186–194, 1996
6. Del Papa N, Guidali L, Sironi M, *et al*: Anti-endothelial cell IgG antibodies from patients with Wegener's granulomatosis binds to human endothelial cell in vitro and induce adhesion molecule expression and cytokine secretion cells. *Arthritis Rheum* 39:758–766, 1996
7. Damianovich M, Gilburd B, George J, *et al*: Pathogenic role of anti-endothelial cell antibodies in vasculitis. An idiotypic experimental model. *J Immunol* 156:4946–4951, 1996
8. Chan TM, Frampton G, Jayne DR, *et al*: Clinical significance of anti-endothelial cell antibodies in systemic vasculitis: A longitudinal study comparing anti-endothelial cell antibodies and antineutrophil cytoplasm antibodies. *Am J Kidney Dis* 22:387–392, 1993
9. Page A, Rose M, Yacoub M, Pigott R: Antigenic heterogeneity of vascular endothelium. *Am J Pathol* 141:673–683, 1992
10. Tsukada N, Tanaka Y, Miyagi K, *et al*: Autoantibodies to each protein fraction extracted from cerebral endothelial cell membrane in the sera of patients with multiple sclerosis. *J Neurol* 24:41–46, 1989
11. Trojano M, Defazio G, Ricchiati F, *et al*: Serum IgG to brain microvascular endothelial cells in multiple sclerosis. *J Neurol Sci* 143:107–113, 1996
12. Praprotnik S, Blank M, Levy Y, *et al*: Anti-endothelial cell antibodies from patients with thrombotic thrombocytopenic purpura specifically activate small vessel endothelial cells. *Int Immunol* 13:203–210, 2001
13. Blank M, Krause I, Goldkorn T, *et al*: Monoclonal anti-endothelial cell antibodies from a patient with Takayasu arteritis activate endothelial cells from large vessels. *Arthritis Rheum* 42:1421–1432, 1999
14. Jeannette CJ, Falk RJ, Andrassy K, *et al*: Nomenclature of systemic vasculitides: Proposal of an international consensus conference. *Arthritis Rheum* 37:187–192, 1994
15. Luqmani R, Bacon PA, Moots RJ, *et al*: Birmingham vasculitis activity score (BVAS) in systemic necrotizing vasculitis. *Q J Med* 87:671–678, 1994
16. Daneker GW, Lund SA, Caughman SW, *et al*: Culture and characterization of sinusoidal endothelial cells isolated from human liver. *In vitro Cell Dev Biol* 34:370–377, 1998
17. Bo X, Broomé U, Uzunel M, *et al*: Capillarization of hepatic sinusoid by liver endothelial cell-reactive autoantibodies in patients with cirrhosis and chronic hepatitis. *Am J Pathol* 163:1275–1289, 2003
18. Bo X, Broomé U, Remberger M, Sumitran-Holgersson S: Tumor necrosis factor α impairs function of liver derived lymphocytes and natural killer cells in patients with primary sclerosing cholangitis. *Gut* 49:131–141, 2001
19. Sumitran-Karuppan S, Lindholm A, Möller E: Fewer acute rejection episodes and improved outcome in kidney transplanted patients with changed selection of criteria based on cross-matching? *Transplantation* 53:666–673, 1992
20. Sumitran-Karuppan S, Möller E: Specific inhibition of HLA class I and II antibodies by soluble antigens—A method for the identification of antibody specificity in serum from alloimmunized individuals. *Transplantation* 58:713–719, 1994
21. Lindquist KJ, Osterland CK: Human antibodies to vascular endothelium. *Clin Exp Immunol* 9:753–760, 1971
22. Tan EM, Pearson CM: Rheumatic disease sera reactive with capillaries in the mouse kidney. *Arthritis Rheum* 15:23–28, 1972
23. Hashemi S, Smith CD, Izaguirre CA: Anti-endothelial cell antibodies: Detection and characterization using a cellular enzyme-linked immunosorbent assay. *J Lab Clin Med* 109:434–440, 1987
24. Rosenbaum J, Pottinger BE, Woo P, *et al*: Measurement and characterisation of circulating anti-endothelial cell IgG in connective tissue disease. *Clin Exp Immunol* 72:450–456, 1988
25. Westphal JR, Boerbooms AMT, Schalkwijk CJM, *et al*: Anti-EC antibodies in sera of patients with autoimmune disease: Comparison between ELISA and FACS analysis. *Clin Exp Immunol* 96:444–449, 1994
26. Kellenberger E, Johansen R, Maeder M, *et al*: Artefacts and morphological changes during chemical fixation. *J Microscopy* 168:181–201, 1992
27. Drover S, Marshall WH: Glutaraldehyde fixation of target cells to plastic for ELISA assays of monoclonal anti-HLA antibodies produces artefacts. *J Immunol Methods* 90:275–281, 1986
28. Perrot JY, Krief P, Julien C, *et al*: Screening monoclonal antibodies against cell-surface antigens. High frequency of natural antibodies. *Scand J Immunol* 23:623–629, 1986
29. Ribatti D, Nico B, Vacca A, *et al*: Endothelial cell heterogeneity and organ specificity. *J Hematotherapy Stem Cell Res* 11:81–90, 2002
30. Aird WC: Endothelial cell heterogeneity. *Crit Care Med* 31:S221–S230, 2003
31. Stastny P: Endothelial-monocyte antigens. *Transplant Proc* 12 (3 Suppl 1):32–36, 1980
32. Brasile L, Rodman E, Shield CFD, *et al*: The association of anti-vascular endothelial cell antibody with hyperacute rejection: A case report. *Surgery* 99:637–640, 1986
33. Cerilli J, Brasile L: Endothelial cell alloantigens. *Transplant Proc* 12 (3 Suppl 1):37–42, 1980
34. Meroni PL, Del Papa N, Raschi E, *et al*: Is there any pathogenic role for the anti-endothelial cell antibodies (AECA) in autoimmune vasculitis. *J Biol Regul Homeost Agents* 11:127–132, 1997
35. Leung DY, Moake JL, Havens PL, *et al*: Lytic anti-endothelial cell antibodies in haemolytic-uraemic syndrome. *Lancet* 2:183–186, 1988
36. Yamaoka J, Kabashima K, Kawanishi M, *et al*: Cytotoxicity of IFN-gamma and TNF-alpha for vascular endothelial cell is mediated by nitric oxide. *Biochem Biophy Res Com* 291:780–786, 2002
37. Holler N, Zaru R, Micheau O, *et al*: Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. *Nat Immunol* 1:489–495, 2000